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I constructed a substrate phage display library displaying randomized hexamer amino acid sequences to explore the possibility of profiling protease activity in non-metastatic breast tumor and metastatic breast tumor. This technique was first applied to two breast cancer implicated and closely related metalloproteinase, MMP-2 and MMP-9. Novel substrate specificity was found for MMP-2 vs. MMP-9. These novel substrate sequences can serve as a tool for us to investigate the role of MMP-2 and MMP-9 during breast tumor progression. Moreover, these unique substrate sequences can be used as leads for designing specific metalloproteinase inhibitors to intervene or eradicate breast tumor. Most importantly, the success of defining substrate specificity of MMP-2 and MMP-9 validates the feasibility of profiling protease activity via substrate phage display library technique.

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Introduction:

The purpose of my study is to obtain a collective knowledge of proteases which activity are differentially overexpressed during breast cancer progression using phage display technology. The profile of proteases or "protease fingerprint" will then be validated as a diagnostic tool for predicting breast cancer progression.

Body:

It is well-documented fact that early detection of breast tumors in patients can have significant impact on the survival rate of patients. Therefore, researchers have been actively searching for biomarkers which can be used to predict breast cancer progression. A good biomarker for breast cancer should have protein expression pattern correlates well with either the onset of malignant tumor or the tumor progression. In this fellowship proposal, I suggest that a profile of protease activity can be used as a diagnostic tool to predict breast cancer progression. Proteases capable of modifying extracellular matrix such as matrix metalloproteinase 2,7and 9 have been shown to involve in breast tumor metastasis, invasion, and angiogenesis(Ding, Coombs et al. 1995; Smith, Shi et al. 1995; Ke, Coombs et al. 1997; Coombs, Bergstrom et al. 1998). They are differentially overexpressed either in the stromal cells surrounding the tumor cells or in the tumor cells (Liotta 1992; Davies, Brown et al. 1993; Stetler-Stevenson, Liotta et al. 1993; Wang, Fu et al. 1994; DeClerck, Imren et al. 1997; Noel, Gilles et al. 1997). However, since these proteases can exist as three forms: inactive form with the attachment of the pro-domain, active form, inactive form complexed with endogenous inhibitors. Therefore, by monitoring the actual protease activity in tumor progression, we can grasp the alteration of tumor development and compile the information obtained into a predictable pattern for tumor progression. To approach this goal, I offered three aims in this proposal.

Aim#1: Construct a substrate phage display library to use as a tool for studying protease fingerprint.

It has been well established that the true assessment of the activity a protease is by measuring the hydrolysis of a peptide or protein substrate by the protease. Therefore, a substrate-based hydrolysis assay will be ideal for monitoring protease activity present during breast tumor development. Furthermore, several different proteases have been reported to involve in breast tumor progression and more proteases might yet to be discovered to involve in different processes of tumor progression, large amount of substrate sequences must be present in the assay to screen for protease activity in vivo to ensure a fair and unbiased assessment for proteases with different substrate preferences and perhaps novel proteases. In aim 1, I hypothesized that the substrate phage display library technique is an ideal assay that can be used to assess the activity of multiple proteases in vivo. Several substrate phage display libraries has been constructed by other researchers and used to extract information of preferred substrate sequences of disease related proteases such as plasminogen activators, thrombin, and MMP-7 (Ding, Coombs et al. 1995; Smith, Shi et al. 1995; Ke, Coombs et al. 1997; Coombs, Bergstrom et al. 1998). Moreover, large amount of randomized sequences can be obtained from a substrate display library (> $1x10^8$ sequences per library) to ensure a good representation of substrate sequences to detect the activity of multiple proteases.

To use as a tool of investigating protease activity, I construct a substrate phage display library which displays randomized hexameric inserts on the surface of phage. It is a modified version of polyvalent phage display system previously constructed by Scott and Smith (Smith 1985; Scott 1992; Smith 1993). The randomized hexameric sequences would mimic the peptide substrates and fit into the protease enzymatic pocket. In addition, I engineered a FLAG epitope tag at the N-terminal of the gIIIp-hexamer fusion protein to facilitate the separation of phages displayed preferred substrate sequences from other phages after each round of protease substrate selection. When a protease is exposed to the preferred substrate sequence displayed on the surface of phage, it will cleave the substrate sequence and liberate both the substrate and the FLAG epitope tag from the surface of phage. Subsequently, phages display protease preferred substrate sequences could be enriched by immuno-precipitaton using a monoclonal anti-FLAG antibody and further amplified. A graphic illustration of this procedure can be found in figure 1 in the next page.

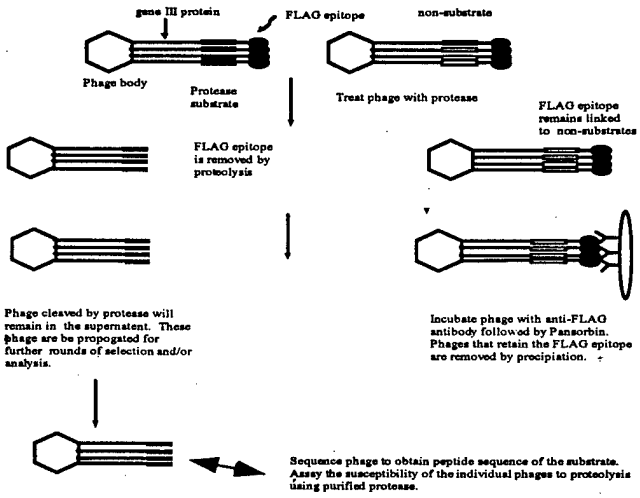

Figure 1. Scheme for selection of substrates by phage display technique.

Three substrate phage display libraries described above were constructed separately and represented by $1.8x10^8$, $2.4x10^8$, and $6.8x10^8$ independent clones. I have confirmed the expression of FLAG epitope by Western Blot analysis, and a monoclonal antibody against the FLAG epitope can be used to immuno-precipitate phage. These libraries are at least as complex as any substrate phage libraries reported so far (Ding, Coombs et al. 1995; Smith, Shi et al. 1995; Ke, Coombs et al. 1997; Coombs, Bergstrom et al. 1998). This level of complexity is crucial to ensure 90% confidence level of including all possible combinations of randomized hexamer sequences. In which case, I am confident that these libraries can be used to explore substrates selectivity among different proteases.

Aim#2: Characterize profile of protease activity present in the blood of mice bearing non-metastatic breast cancer tumors and mice bearing metastatic breast cancer tumors.

Matrix metalloproteinases have been shown to play important roles in breast tumor invasion, metastatsis, and angiogenesis (Liotta 1992; Stetler-Stevenson, Liotta et al. 1993; Noel, Gilles et al. 1997). Particularly, matrix metalloproteinase 2 and 9 have been studied extensively for their direct involvement in all of the processes required during tumor progression (Festuccia, Bologna et al. 1996; Sier, Kubben et al. 1996; Stearns and Stearns 1996; Stearns and Stearns 1996; Gohji, Fujimoto et al. 1998; Itoh, Ito et al. 1998; Kurizaki, Toi et al. 1998; Leppert, Ford et al. 1998; Moses, Wiederschain et al. 1998; Yu and Stamenkovic 2000). Like other matrix metalloproteinases, MMP-2 and MMP-9 are capable of cleaving extracellular matrix components such as collagen and laminin. Increased gene and protein expression of both proteases has been reported to correlate with tumor invasion and metastasis (Brooks, Stromblad et al. 1996; Festuccia, Bologna et al. 1996; Sier, Kubben et al. 1996; Stearns and Stearns 1996; Stearns and Stearns 1996; Gohji, Fujimoto et al. 1998; Kurizaki, Toi et al. 1998; Moses, Wiederschain et al. 1998). Also, from the immunohistological studies, MMP-2 and MMP-9 were found to localize in the tumor tissue (Brooks, Stromblad et al. 1996; Stearns and Stearns 1996; Stearns and Stearns 1996). To illustrate how these two proteases can be utilized by tumor cells, a mechanism of cell-surface association and activation for MMP-2 has been proposed to be used by tumor cells for invasion and metastatsis (Strongin, Marmer et al. 1993; Strongin, Collier et al. 1995). As a validation of the substrate phage display library I constructed, I chose to define substrate specificity of MMP-2 and MMP-9 using purified proteins. Several advances could come from the study of substrate specificity. First, specific substrate sequences preferred by one protease over the other will help us distinguish functions of these two proteases. Unknown physiological substrates can potentially be revealed by defining the substrate preferences of each protease. Furthermore, the selected specific substrate sequences can serve as lead compounds for therapeutic and diagnostic development.

Since the hemopexin domain of both proteases have been shown to influence only the cell-surface association but not catalytic function of the protease (Murphy, Willenbrock et al. 1992), I chose to conduct the substrate specificity comparison with only the purified MMP-2 and MMP-9 catalytic domain. Embryotic kidney 293 cells were transfected separately with cDNA of either truncated MMP-2 or MMP-9 which includes only the pro-domain and truncated catalytic domain (no fibronectin-like domain) as described previously (Murphy, Willenbrock et al. 1992). Then, MMP-2 and MMP-9 protein were purified from conditioned media using gelatin affinity column as reported previously (Hipps, Hembry et al. 1991). The activity and purity of the purified MMP-2 and MMP-9 were verified by gelatin zymography and active site titration using a synthetic metalloproteinase inhibitor, ilomastat (Galardy, Cassabonne et al. 1994; Galardy, Grobelny et al. 1994). To select for MMP-2 substrates, $2x10^{10}$ phage from the initial library consisting of $2.4x10^{8}$ independent clones were incubated with 10ug/ml of purified MMP-2 at 37°C. Concurrently, a control reaction of $2x10^{10}$ phage with no protease was set up and incubated at the same condition as the selection reaction. Following the protease treatment, 100ug of monoclonal anti-FLAG antibody were added to both reactions, and the reactions were incubated at 4°C over night with rocking. Following the overnight incubation, pansorbin, which is inactivated bacterial cells expressing high concentration of protein A on the surface, was added to both reactions to precipitate the FLAG:IgG complexes, leaving the phage with cleaved substrate sequences in solution. The remaining phage, which carries MMP-2 preferred substrate sequences, was then amplified by infecting K91 bacteria and used for the subsequent round of selection.

For facilitate the screening of phage clones that carry protease preferred substrate sequences, a substrate phage ELISA was developed to rapidly identify desired phage clones. This assay has two major advantages. First, it eliminates the time consuming and tedious phage purification procedures by capturing the phage from the over night phage culture. Second, the readout of this assay yield a semi-quantitative indication of the rate of hydrolysis by the protease for individual sequences. Following two rounds of selection with purified MMP-2, I perfomed substrate phage ELISA to identify and characterize the ability of individual phage clones to be cleaved by MMP-2 as described in figure 2 below.

The substrate phage ELISA is carried out in 96-well microtiter plate format. A 96 well plate is coated with an anti-phage (gVIIIp) antibody. The wells are then incubated with 150ul supernatant from the over night culture of each tested phage clone. Four wells are coated with the same test phage clone: two wells as control with no protease, and two wells are for protease treatment. After two hours of phage binding at 4°C, 5ug/ml of MMP-2 were added to the designated wells and the plate is incubated at 37°C. In protease treatment reaction, MMP-2 will cleave the substrate and liberate the FLAG epitope from the phage. After protease incubation, the loss of FLAG epitope is detected by an anti-FLAG polyclonal antibody, followed by HRP-conjugated secondary antibody. Protease cleavage is indicated by the loss of FLAG epitope. This assay not only confirms that a peptide is a substrate, it also establishes a relative rate of hydrolysis between the substrates, that is the rate at which each substrate is hydrolyzed. In addition, it also allows me to test many potential substrates in one assay. Up to 100 substrates can easily be tested in one day.

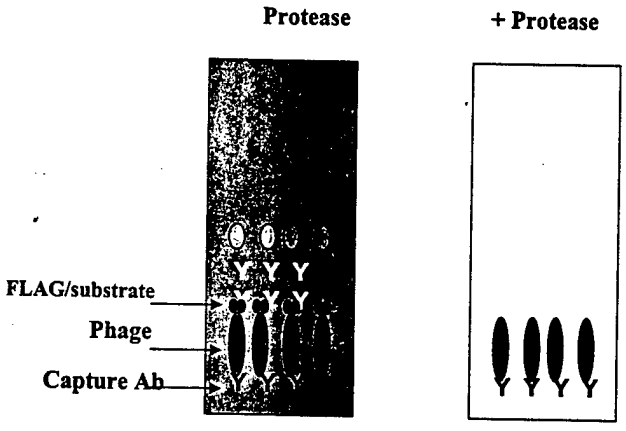

Figure 2. Scheme for substrate phage ELISA.

A number of phage clones were selected by substrate phage ELISA with the loss of greater than 10% of the FLAG epitope due to the cleavage of substrate by MMP-2. DNA sequence analysis was performed for the selected phage clones to obtain the substrate sequences. Subsequent substrate sequence analysis revealed that three major classes of peptide substrates for MMP-2 were observed and summarized in table 1 in the next page. First major class represents a previously defined substrate preference for MMP-2 and MMP-9. Second and third classes represent two families of previously undefined substrates that could potentially distinguish MMP-2 from MMP-9 based on the data of substrate ELISA.

MMP-2 substrate classes	
Class 1: P-X-X-Hydrophobic amino acid	Previously defined substrates
Class 2: L-X-X-Hydrophobic amino acid	Novel substrates
Class 3: S-X-L	Novel undefined substrates

Table 1. Summary of three classes of MMP-2 peptide substrates.

One peptide from the PXX(hydrophobic amino acids) family, A-3, and one peptide from the LXX(hydrophobic amino acids), A49, were synthesized and their Kcat/Km values were determined for both MMP-2 and MMP-9. To determine the Kcat and Km value, I used a fluorescamine based assay(Fields, Van Wart et al. 1987). Fluorescamine is intrinsically nonfluorescent but reacts in milliseconds with primary aliphatic amines, including peptides and proteins, to yield a fluorescent derivative. After incubation of protease and peptide, the peptide is cleaved and the cleavage can be detected by addition of fluorescamine and read by a fluorescent microplate reader. Four different concentrations of each peptide were used to obtain the double reciprocal plot of initial velocity and substrate concentration. Km and Kcat values were then determined from the graph. The Kcat/Km values are listed in table 2 below.

Peptide	Sequence	MMP-2			MMP-9		
		Kcat/Km ($M^{-1}S^{-1}$)	Kcat (S^{-1})	Km (mM)	Kcat/Km ($M^{-1}S^{-1}$)	Kcat (S^{-1})	Km (mM)
A-3	S G A K **P** R A↓ **L**	18414.6	3.02	0.164	39591.8	2.91	0.074
A49	S G L R **L** A A↓ **I**	4208.3	1.01	0.24	270	*	*

Interestingly, the kinetic data of these two peptides confirmed the substrate ELISA result. This is first time that a MMP-2 specific peptide substrate sequence is identified. These MMP-2 specific substrate sequences could potentially be used as lead compounds for designing specific metalloproteinase inhibitors. Also, these unique sequences can be modified and then use as a tool to explore the physiological roles of MMP-2 and MMP-9 by discovering new physiological substrates in vivo. Furthermore, being able to define the substrate specificity of these two structurally and functionally related proteases would validate my hypothesis that the substrate phage display library can be used for profiling of different protease activity.

Future plans:

Synthesis of more MMP-2 specific peptides from both class 2 and class 3 MMP-2 substrate sequence is in progress. Their Kcat/Km values will be determined for both MMP-2 and MMP-9 to provide further confirmation of substrate specificity. Afterwards, I will launch to develop the method of protease profiling in vivo as described in aim#2. The mouse breast cancer model will be developed to obtain blood samples. Subsequently, I will select protease substrates using blood samples from normal mice, mice bearing non-metastatic tumor, and mice bearing metastatic tumor.

Key research accomplishments:

- I constructed three substrate phage display libraries with the highest complexity ever reported in the literature.
- I found new substrate sequences that are preferred by MMP-2 over MMP-9. These substrate sequences have therapeutic values because they can potentially be used as lead compounds for designing specific inhibitors.

Reportable outcomes:

These data were presented in the Annual Symposium of the Molecular Pathology Graduate Program of UCSD School of Medicine in June 22, 2000.

Conclusions/Implications:

- New families of MMP-2 substrates have been discovered. These substrates have selectivity for MMP-2 over MMP-9
- Substrate specificity appears to be governed by the S3 constituent of the substrate
- Our findings suggest many important substrates for MMP-2 may have been overlooked. These could clarify the biological role of MMP-2 (and MMP-9).

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